

# CB<sub>1</sub> cannabinoid receptors are involved in neuroleptic-induced enhancement of brain neurotensin

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## ARTICLE INFO

*Article type:*  
Original article

*Article history:*  
Received: Jun 5, 2013  
Accepted: Nov 6, 2013

*Keywords:*  
Amisulpride  
Brain  
CB<sub>1</sub> receptors  
Fluphenazine  
Neurotensin  
Rat

## ABSTRACT

**Objective(s):** Targeting the neuropeptide systems has been shown to be useful for the development of more effective antipsychotic drugs. Neurotensin, an endogenous neuropeptide, appears to be involved in the mechanism of action of antipsychotics. However, the available data provide conflicting results and the mechanism(s) by which antipsychotics affect brain neurotensin neurotransmission have not been identified. Therefore, we aimed to investigate the effects of fluphenazine and amisulpride on brain regional contents of neurotensin considering the role of cannabinoid CB<sub>1</sub> receptors which interact with neurotensin neurotransmission.

**Materials and Methods:** Fluphenazine (0.5, 1, and 3 mg/kg) or amisulpride (3, 5, and 10 mg/kg) were administered intraperitoneally to male Wistar rats either for one day or 28 consecutive days. Twenty four hours after the last injection of drug or vehicle, neurotensin contents were determined in the mesocorticolimbic and nigrostriatal dopamine regions by radioimmunoassay. In the case of any significant change, the effect of pre-treatment with CB<sub>1</sub> receptor antagonist, AM251 was investigated.

**Results:** Chronic, but not acute, treatment with the highest dose of fluphenazine or amisulpride resulted in significant enhancement of neurotensin contents in the prefrontal cortex and nucleus accumbens. Fluphenazine also elevated neurotensin levels in the anterior and posterior caudate nuclei and substantia nigra. Neither amisulpride nor fluphenazine affected neurotensin contents in the amygdala or hippocampus. Pre-treatment with AM251 (3 mg/kg) prevented the neuroleptic-induced elevation of neurotensin. AM251 showed no effect by itself.

**Conclusion:** The brain neurotensin under the regulatory action of CB<sub>1</sub> receptors is involved in the effects of amisulpride and fluphenazine.

## ► Please cite this paper as:

Hassanzadeh P, Rostami F. CB<sub>1</sub> cannabinoid receptors are involved in neuroleptic-induced enhancement of brain neurotensin. Iran J Basic Med Sci; 2014; 17:181-188.

## Introduction

Mental disorders including schizophrenia are widely recognized as a leading cause of the disability worldwide (1). Schizophrenia is a major clinical syndrome which is characterised by multiple signs and symptoms and is associated with complex and heterogeneous manifestations including the emotional, cognitive, and behavioural disturbances. The onset of symptoms often occurs during adolescence or young adulthood and persists throughout the lifetime. Despite the substantial advances in the field of schizophrenia research, the underlying pathophysiology of the disease is still not fully understood. In this sense, several etiological models have been proposed to explain the biological basis of the disease including the neurodevelopmental, neurodegenerative, and cortical or subcortical disconnection models (2). The putative neurotransmitter dopamine is the most implicated neurochemical substrate in the pathogenesis and pharmacotherapy of schizophrenia (3). Meanwhile,

it has been shown that an imbalance between several neurotransmitter systems is involved in the neuropathology of schizophrenia (4). Neurotransmitter systems are usually modulated by neuropeptides (5), therefore, any alteration in neuropeptide transmission may contribute to the pathophysiology of psychiatric disorders including schizophrenia. In this context, targeting the neuropeptide systems might be a promising therapeutic approach against the psychiatric disorders. Neurotensin, a gut-brain neuropeptide which was first isolated from bovine hypothalamus (6), is implicated in various physiologic and pathologic processes and has shown beneficial effects against the inflammatory disorders (7, 8). This endogenous neuropeptide is widely distributed throughout the central nervous system (CNS) of many mammals including humans and regulates glutamate, gamma-aminobutyric acid (GABA) and serotonin pathways (9, 10).

In brain dopaminergic pathways, neurotensin is

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co-localized with dopamine and modulates dopamine transmission. In this respect, neurotensin-dopamine interactions have been demonstrated in several anatomical, behavioural and pharmacological studies (11, 12). Neurotensin appears to be implicated in the pathophysiology and pharmacotherapy of schizophrenia (13, 14). Based on the previous reports, neurotensin content in the cerebrospinal fluid (CSF) of drug-free schizophrenic patients is reduced and there is a correlation between the CSF concentration of neurotensin and the severity of schizophrenia symptoms (15, 16). Schizophrenic patients with low CSF concentrations of neurotensin are usually lithium non-responders and show a greater degree of thought disorders, hallucinations, and impaired behavioural performance (17). In animal experiments, central administration of neurotensin has shown behavioural and biochemical effects similar to those of antipsychotic drugs (18). However, studies investigating the regulatory effects of antipsychotics on brain neurotensin levels have provided conflicting results (19, 20). In addition, the mechanism(s) of the regulatory effects of neuroleptics on brain neurotensin content have not been fully elucidated. Based on this background, we aimed to investigate the implication of neurotensinergic system in the mechanism of action of fluphenazine and amisulpride. Fluphenazine is a typical antipsychotic drug which blocks the postsynaptic dopaminergic D<sub>1</sub> and D<sub>2</sub> receptors in the mesolimbic system and controls the symptoms in schizophrenia, dementia, agitation, and manic phases of bipolar disorder. The atypical antipsychotic, amisulpride, was initially developed as a selective D<sub>2</sub> and D<sub>3</sub> receptor antagonist for the treatment of schizophrenia, meanwhile, it has shown antidepressant effect via the antagonism at 5-HT<sub>7a</sub> receptors (14, 20).

In order to have a mechanistic approach, we focused on the role of the endocannabinoid signalling in the potential regulatory effects of amisulpride or fluphenazine on brain neurotensin. In recent years, the endocannabinoid system and its regulatory functions in both central and peripheral nervous systems have attracted a growing interest. This ubiquitous signalling system is engaged in a plethora of physiological functions (21) and pathophysiology or treatment of depression (22). Moreover, the endocannabinoid system is involved in the mechanism of action of various psychotropic agents (23-27). According to Rodríguez-Gaztelumendi *et al*, cannabinoid CB<sub>1</sub> receptors and the enzymes involved in the synthesis and degradation of endocannabinoid ligands are located in the brain regions crucial for the emotionality and stress regulation (28). Interestingly, the endocannabinoid system interacts with both neurotensinergic and dopaminergic systems (29, 30). Based on a recent study, cannabinoid CB<sub>1</sub> receptors mediate the gastroprotective effect of neurotensin (31).

Therefore, it may be reasonable to speculate that CB<sub>1</sub> receptors are involved in the regulatory effects of antipsychotic drugs on brain neurotensin content.

## Materials and Methods

### Animals

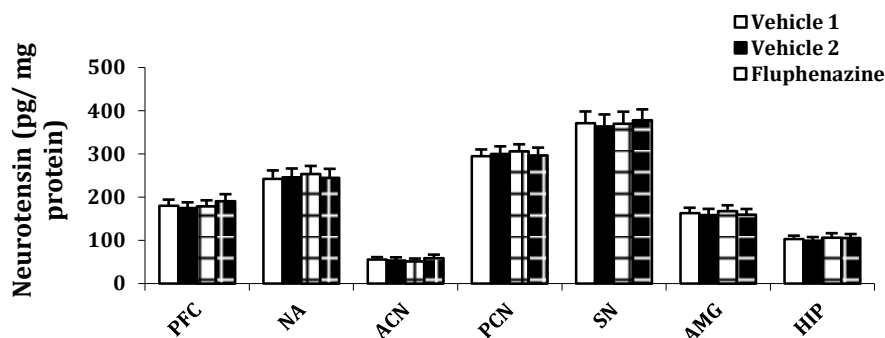
Male Wistar rats weighing 220-250 g were obtained from Pasteur Institute of Iran, Tehran, Iran and housed three per cage under controlled conditions of temperature (22 ± 2°C), humidity (55 ± 10%), and 12 hr light-dark cycle with *ad libitum* access to food and water. Experiments began after at least 1 week of habituation to the housing conditions. All experimental procedures were approved by the Local Ethics Committee.

### Drug treatment

Groups of rats (n=6) received intraperitoneal (IP) injections of 0.5, 1, and 3 mg/kg typical antipsychotic fluphenazine dihydrochloride (Sigma-Aldrich, Germany) (32) which was dissolved in 0.9% saline or 3, 5, and 10 mg/kg atypical antipsychotic amisulpride (Sigma-Aldrich, Germany) (33) dissolved in 70% ethanol and 0.9% saline (3:7) for either one day or 28 consecutive days. Animals in control groups received the equivalent amount of vehicle (n=6). In the case of any significant alteration in brain neurotensin content due to the administration of antipsychotics, the CB<sub>1</sub> receptor antagonist AM251 (Tocris Bioscience, UK) was dissolved in Tween 80 (Sigma Aldrich, Germany), dimethyl sulfoxide (Sigma Aldrich, Germany), and 0.9% saline (1:1:8) and injected IP at the doses of 1, 2 and 3 mg/kg (34, 35) either alone or 30 min before the administration of antipsychotic (n=6). Drugs were injected between 9:00 and 10:00 a.m. at a total volume of 1 ml/kg.

### Dissection of the brain regions

Twenty four hours after the last injection of drug or vehicle (36), animals were sacrificed by exposing their heads to a focused beam of microwave irradiation for 3.5 sec. This method has the advantage over the decapitation in minimizing post-mortem changes of neurotensin content by rapid thermal inactivation of tissue enzymatic activities (37). Animals were sacrificed between 10:00 a.m. and 1:00 p.m. in order to minimize the variability due to diurnal fluctuations. The brain of each animal was quickly and carefully removed from the skull and dissected on ice into the prefrontal cortex, nucleus accumbens, anterior and posterior caudate nuclei, substantia nigra, amygdala and hippocampus (38, 39). Tissue samples were immediately frozen on dry ice, weighed and kept in 500 µl polyethylene microcentrifuge tubes and stored at -70°C until extracted.



**Figure 1.** Acute administration of fluphenazine or amisulpride does not alter brain regional levels of neurotensin. Vehicles 1 and 2 are related to fluphenazine and amisulpride, respectively. Data are expressed as mean  $\pm$  SEM of  $n=6$ /group  
PFC: Prefrontal cortex, NA: Nucleus accumbens, ACN: Anterior caudate nucleus, PCN: Posterior caudate nucleus, SN: Substantia nigra, AMG: Amygdala, HIP: Hippocampus

### Quantification of neurotensin

The concentration of neurotensin in the tissue supernatants was determined by a sensitive and specific radioimmunoassay (40). Briefly, each brain region was homogenized by ultrasonic disruption in 500  $\mu$ l of ice-cold 1 M HCl and centrifuged at  $10^4$  g for 15 min at 4°C. The supernatant was transferred to a microcentrifuge tube and vortexed, and duplicate aliquots of 100  $\mu$ l were transferred to borosilicate glass tubes and stored at -70°C. Upon the assay performance, the frozen aliquots were lyophilized, reconstituted in the assay buffer including 10 mM  $\text{NaH}_2\text{PO}_4$ , 0.15 M NaCl, 0.01%  $\text{NaN}_3$ , 0.1% gelatin, 2.5 mM EDTA, and 0.05% Triton X-100 adjusted to pH 7.6 with NaOH. Neurotensin antiserum (Peninsula Laboratories, UK) which is directed toward the middle portion of the neurotensin molecule, was used at a final dilution of 1:13,000 that provides 30% zero binding of the  $^{125}\text{I}$ -labeled neurotensin (Amersham International, UK). Synthetic neurotensin (Peninsula Laboratories, UK) was considered as a standard. Free and antibody-bound neurotensin were separated by 50  $\mu$ l goat anti-rabbit antibody (Peninsula Laboratories, UK) as the secondary antibody. Samples were left for 30 min at room temperature, then, the reaction was blocked with 1 ml distilled water. After centrifugation at

3000 g for 20 min at 4°C, the supernatants were decanted and the radioactivity in the pellets was determined using a gamma counter (LKB Wallac, Finland) with a 2min/tube counting time and a 67% counting efficiency. The assay has a sensitivity of 1.25 pg/tube and an  $\text{IC}_{50}$  of 80 pg/tube. Tissue pellets were resuspended in 1M NaOH by sonication and assayed for total protein (41). The concentration of neurotensin is expressed as pg of neurotensin per mg of protein.

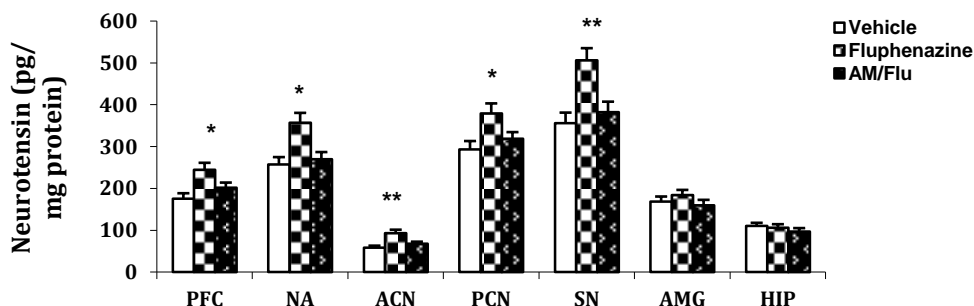
### Statistics

Data were analysed by analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Data are presented as mean  $\pm$  SEM (six animals per group). The level of significance was set at  $P < 0.05$ .

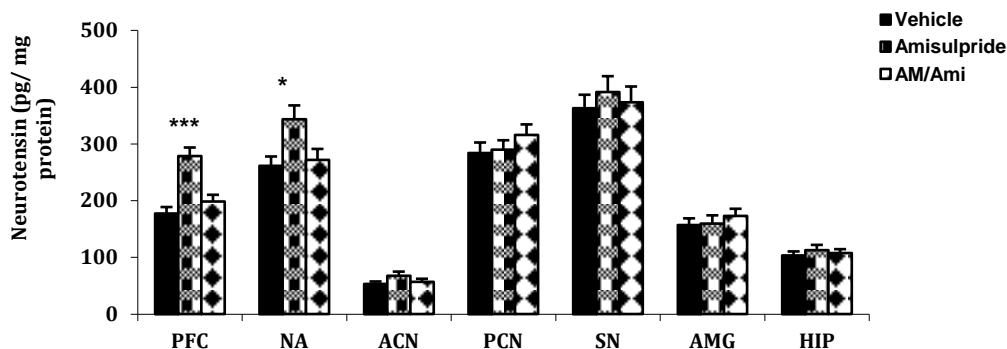
### Results

#### The effects of acute administration of fluphenazine or amisulpride on brain regional levels of neurotensin

Single injection of 0.5 mg/kg fluphenazine or 3 mg/kg amisulpride did not affect brain neurotensin content as compared to the corresponding vehicles (Figure 1,  $P > 0.05$ ). Acute administration of the higher doses of drugs did not result in a remarkable effect (not shown).



**Figure 2.** The effect of chronic treatment with fluphenazine on brain neurotensin content and the role of AM251 in this regard. 28-day treatment with fluphenazine 3 mg/kg resulted in a brain region-specific enhancement of neurotensin contents. This was prevented due to the daily pre-application of the  $\text{CB}_1$  receptor antagonist AM251 (3 mg/kg). Data represent mean  $\pm$  SEM of  $n=6$ /group. \* $P < 0.05$ , \*\* $P < 0.01$ . (AM/Flu: injection of AM251 30 min before the exposure to fluphenazine). PFC: Prefrontal cortex, NA: Nucleus accumbens, ACN: Anterior caudate nucleus, PCN: Posterior caudate nucleus, SN: Substantia nigra, AMG: Amygdala, HIP: Hippocampus



**Figure 3.** The effect of chronic administration of amisulpride on brain neurotensin levels and the role of AM251 in this regard. Four-week daily administration of amisulpride 10 mg/kg elevated neurotensin level in a brain region-specific fashion that was prevented by AM251 (3 mg/kg) pre-treatment. Data represent mean ± SEM of n=6/group. \* $P<0.05$ , \*\*\* $P<0.001$  (AM/Ami: injection of AM251 30 min prior to the administration of amisulpride) PFC: Prefrontal cortex, NA: Nucleus accumbens, ACN: Anterior caudate nucleus, PCN: Posterior caudate nucleus, SN: Substantia nigra, AMG: Amygdala, HIP: Hippocampus

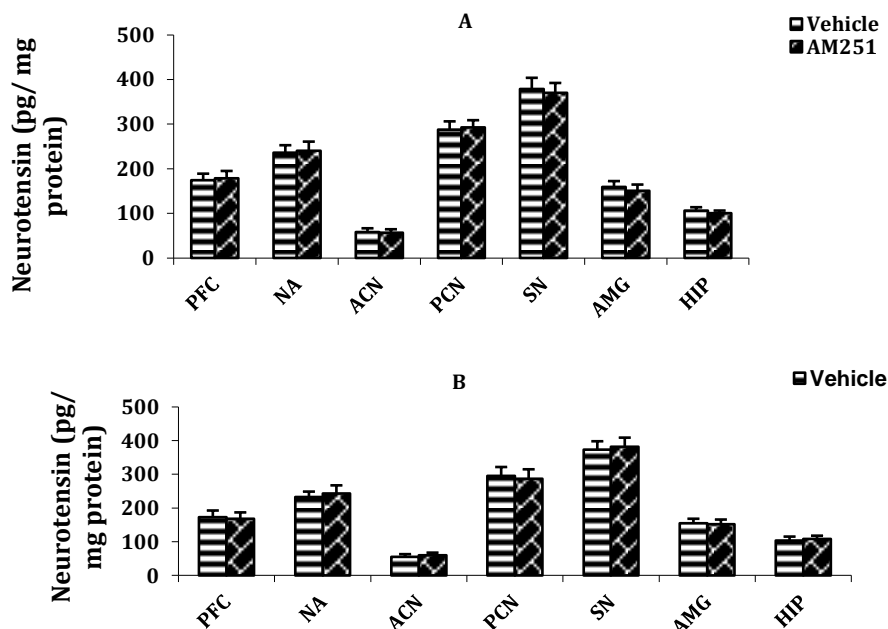
**The effect of chronic treatment with fluphenazine on brain neurotensin content and the role of AM251 in this regard**

Twenty eight-day treatment with the lower doses of fluphenazine did not alter neurotensin concentration in any brain region examined (not shown). As shown in Figure 2, 24 hr after the last injection of the highest dose of fluphenazine, *post hoc* comparisons revealed a significant elevation of neurotensin content in the prefrontal cortex ( $P<0.05$ ) and nucleus accumbens ( $P<0.05$ ). Furthermore, fluphenazine elevated neurotensin levels in the anterior and posterior caudate nuclei

( $P<0.01$  and  $P<0.05$ ) and substantia nigra ( $P<0.01$ ). Fluphenazine did not alter neurotensin content in the amygdala or hippocampus ( $P>0.05$ ). Daily pre-treatment of 1 or 2 mg/kg AM251 did not affect fluphenazine-induced elevation of neurotensin (not shown), while, 3 mg/kg AM251 showed a preventive effect in this regard (Figure 2,  $P>0.05$ ).

**The effect of chronic treatment with amisulpride on brain neurotensin content and the role of AM251 in this regard**

Four-week daily administration of the lower doses of amisulpride did not affect neurotensin



**Figure 4.** AM251 alone does not affect brain regional levels of neurotensin. A: Single injection of 3 mg/kg AM251, B: 28-day treatment with 3 mg/kg AM251. Data represent means ± SEM of n=6/group PFC: Prefrontal cortex, NA: Nucleus accumbens, ACN: Anterior caudate nucleus, PCN: Posterior caudate nucleus, SN: Substantia nigra, AMG: Amygdala, HIP: Hippocampus



content in any brain region investigated (not shown). As shown in Figure 3, 24 hr after the last injection of the highest dose of amisulpride, a significant enhancement of neurotensin levels was observed in the prefrontal cortex ( $P<0.001$ ) and nucleus accumbens ( $P<0.05$ ). Amisulpride had no remarkable effects on other brain regions investigated ( $P>0.05$ ). Daily pre-treatment with 1 or 2 mg/kg AM251 did not affect amisulpride-induced enhancement of neurotensin (not shown), while, 3 mg/kg AM251 showed a preventive effect in this regard (Figure 3,  $P>0.05$ ).

#### **The effect of AM251 on brain regional content of neurotensin**

Acute or 28-day treatment with AM251 (3 mg/kg) alone did not alter neurotensin content in any brain region analyzed (Figure 4A and B,  $P>0.05$ ).

### **Discussion**

During the last decades, targeting the neuropeptide systems which are capable of regulating several neurotransmitter systems, has been the focus of intense research in order to develop more effective antipsychotic drugs. In this context, neurotensinergic neurotransmission has attracted a growing interest (13, 14, 19, 20, 36). Based on the neuroleptic effect of neurotensin, it has been suggested that neurotensin inhibits dopaminergic neurotransmission in dopamine-rich regions of brain such as the prefrontal cortex and nucleus accumbens (18). In addition to the direct action, neurotensin can indirectly affect dopaminergic transmission through its association with other neurotransmitter systems including the glutamatergic, GABAergic, and serotonergic systems. Enhancement of GABA release due to the activation of neurotensin receptors may result in the reduction of dopamine release via the activation of GABA receptors located on dopamine terminals (11, 12, 29). As mentioned before, antipsychotic drugs may attenuate dopamine neurotransmission through the elevation of neurotensin. Meanwhile, the previously conducted studies represent different, sometimes conflicting, data (13, 19, 20, 36). In the present study, we have investigated the potential implication of neurotensin in the mechanism of action of the typical and atypical antipsychotic drugs, fluphenazine and amisulpride. As shown in Figure 1, neurotensin is distributed in brain regions which are associated with the pathophysiology of schizophrenia and acute administration of fluphenazine or amisulpride did not affect neurotensin content in these parts. However, four-week daily administration of these neuroleptics increased neurotensin levels in a dose-dependent and brain region-specific fashion (Figures 2 and 3). This finding may be in accordance with the delay in the onset of clinical efficacy after the

treatment with neuroleptics. Moreover, enhancement of neurotensin content in dopamine-rich brain regions following chronic administration of fluphenazine or amisulpride may represent a compensatory mechanism as part of the adaptive response to the prolonged dopamine receptor blockade. According to Adachi *et al*, neurotensin may bind to dopamine leading to the reduction of its availability (42). Meanwhile, the interaction of neurotensin with other neurotransmitter systems should not be excluded.

Chronic treatment with fluphenazine or amisulpride elevated neurotensin content in the prefrontal cortex and nucleus accumbens (Figures 2 and 3). These findings provide evidence for a role of increased corticolimbic neurotensin neurotransmission in the mechanism of action of both of these typical and atypical antipsychotics. It appears that the neurochemical effects shared by fluphenazine and amisulpride may mediate their therapeutic efficacies. As previously reported, the deficits in neurotensin neurotransmission are associated with functional and behavioural disruptions similar to those seen in schizophrenic patients and may also be linked to deficits in sensorimotor gating (43). Therefore, normalization of neurotensin neurotransmission following antipsychotic therapy may cause a recovery of such deficits.

As demonstrated in Figures 2 and 3, fluphenazine, but not amisulpride, elevated neurotensin content in the nigrostriatal regions, indicating that the therapeutic effects of amisulpride, at least in part, may be due to its effects on the neurotensin neurotransmission in the mesolimbic system but its actions in the nigrostriatal regions are much less potent. The latter finding also suggests that dopamine projections which terminate in the nigrostriatal regions are affected differentially by fluphenazine and amisulpride consistent with their differential liabilities to induce extrapyramidal side effects. The nigrostriatal regions control planning and execution of motor behaviours (44) and may be associated with the extrapyramidal side effects which usually occur following the treatment with typical antipsychotics. As previously reported, activation of nigral neurotensin receptors contributes to the inhibition of the nigrothalamic GABAergic pathway (45). This may result in the disinhibition of the excitatory glutamatergic drive to the motor cortex and justify the extrapyramidal side effects induced by typical antipsychotics including fluphenazine. As a whole, enhancement of neurotensin content in the nigrostriatal and mesocorticolimbic brain regions following chronic treatment with fluphenazine or amisulpride suggests that neurotensinergic pathways convey distinct information to these dopamine-rich regions of brain that may lead to the regulation of different

physiological processes. In this context, neurotensin may be considered as a neuroanatomically-selective neuropeptide which mediate the therapeutic as well as the extrapyramidal side effects of antipsychotics.

Chronic administration of the highest dose of fluphenazine or amisulpride did not affect neurotensin contents in the amygdala and hippocampus (Figures 2 and 3), indicating that neurotensinergic neurotransmission in these brain regions is not involved in the mechanism of action of these antipsychotics. Meanwhile, based on a study conducted by Gruber *et al*, the typical antipsychotic haloperidol elevated neurotensin concentration in the hippocampus (19). Moreover, clozapine in contrast to amisulpride reduced neurotensin content in the prefrontal cortex (36). One likely explanation for the discrepancies between our findings and those of others may be due to the methodological differences such as the animal species, different dissection techniques or the neuroleptic regimens followed. It is possible that the neurotensin system in dopamine-rich brain regions reacts differently in response to dopamine-altering drugs. According to the heterogeneity of antipsychotic drugs such as their different binding affinities, they may regulate the neurotensinergic system by different mechanisms. In this context, typical or atypical antipsychotics do not affect brain neurotensin neurotransmission in a homogenous fashion. Meanwhile, the previously published data and ours might have important implications for the understanding of the functional association between dopamine- and neurotensin-containing cells in the CNS as well as the mechanisms of action of antipsychotic drugs.

The CB<sub>1</sub> receptor antagonist AM251 (3 mg/kg) prevented the neuroleptic-induced enhancement of neurotensin contents via blocking the endogenous cannabinoid activity (Figures 2 and 3). On the other hand, AM251 showed no effects by itself (Figure 4). These findings suggest that fluphenazine and amisulpride affect brain neurotensin neurotransmission under the regulatory drive of CB<sub>1</sub> receptors. We have also shown the implication of CB<sub>1</sub> receptors in the neurotrophic effects of antipsychotic drugs (25). These findings indicate the critical role of the endocannabinoid system in the pathophysiological mechanisms underlying schizophrenia as well as its possible help in leading us toward the development of more effective drugs.

## Conclusion

Our findings indicate that the increased mesolimbic neurotensin contents is implicated in the mechanism of action of both fluphenazine and amisulpride. The differential effects of fluphenazine and amisulpride on neurotensin neurotransmission in the nigrostriatal dopaminergic system may underlie the

differences between the therapeutic profile of these neuroleptics as well as the extrapyramidal side effect liability of fluphenazine. This study shows once again the importance of neurotensin hypothesis in the etiopathogenesis and/or treatment of schizophrenia and argues for the CB<sub>1</sub> receptor-mediated up-regulation of brain neurotensin content by amisulpride or fluphenazine.

## Acknowledgment

This study was funded in part by a grant from Tehran University of Medical Sciences, Tehran, Iran. Authors wish to thank Professor Majid Ghaffarpour, Iranian Center of Neurological Research, Tehran University of Medical Sciences, for fruitful discussion.

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